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RESEARCH PAPER

Inhibition effect of expression of Cu/Zn superoxide dismutase from rice on synthesis of Glutathione in *Saccharomyces cerevisiae*

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Abstract: The expression of a rice Cu/Zn superoxide dismutase (Cu/Zn-SOD) in *Saccharomyces cerevisiae* regulated by GAPDH promoter, involved in the inhibition of endogenous Glutathione (GSH) synthesis, and the competitive expression was detected by constructing the expression vector transferred Cu/Zn-SOD gene into wild-type *S. cerevisiae*. Transcription and expression of the Cu/Zn-SOD gene in *S. cerevisia*were were confirmed by northern blot and SDS-PAGE, respectively, and activity of the Cu/Zn-SOD from crude extracts was enzymatically detected based on the effect of nitroblue tetrazolium (NBT) after running a native polyacrylamide gel. The GSH synthesis was also tested by DTNB (5, 5′-Dithiobis (2-nitrobenzoic acid)) method. Results showed that GSH synthesis was evidently suppressed by the expression of Cu/Zn-SOD gene in both control and heat shock strains. It implied that the expression of the Cu/Zn-SOD gene in *S. cerevisiae* has more potential facility in response to oxidative exposure than that of endogenous GSH, although Cu/Zn-SOD and GSH were both contributed to the function of oxygen radical oxidoreduction.

Keywords: Cu/Zn Superoxide Dismutase; GAPDH promoter; *Saccharomyces cerevisiae*; glutathione

Introduction

All aerobionts are unavoidably subjected to oxidative stress, resulting in consecutive increase in reactive oxygen species (ROS) *in vivo*. These ROS are highly reactive and may damage the cellular organelles such as DNA, proteases and lipids. Cells have developed the oxidoreduction mechanism reducing the excessive ROS from cytoplasm to avoid the above injurious aggression (Derek et al. 1998). Cu/Zn Superoxide Dismutase (Cu/Zn-SOD) is regarded as the first cellular defense enzyme to cope with the reduction of superoxide anion radical and to catalyze the removal of superoxide radicals (Fridovich 1973; Reddy and Venkaiah 1984). Schuessel et al. (2005) reported that impaired Cu/Zn-SOD contributed to the oxidative damage in mice brain. The expression of human Cu/Zn-SOD in *Saccharomyces cerevisiae* may significantly increase the tolerance to oxidative stress (Yoo et al. 1999). All SOD isozymes in various maize lines were consistent in their response to oxidative stress (Jung et al. 2001). The Cu/Zn-SOD from different sources was investigated to share the similar function as oxidative stress resistance (Yoo et al. 1999; Abarca et al. 2001; Mysore et al. 2005; Welch et al. 2006).

Glutathione (GSH) is a small polypeptide compared with Cu/Zn-SOD, being composed of three amino acids, Glutamyl-cysteninyl-glycine. GSH synthesis *in vivo* is directly regulated by two important enzymes, GSH1 and GSH2 (Wachter et al. 2005). Glutathione oxidoreductase EC 1.6.4.2 (GR) is a key enzyme to catalyze the polypeptide from oxidized form (GSSG) to reduced form (GSH) by using nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing cofactor. Tobacco transformed with the glutathione reductase (*gor*) gene encoding GR in *Escherichia coli*, showed mixed results in resistance to oxidative stress, indicating that GSH plays an important role in preventing from oxidative stress (Tang et al. 1994).

In this study, we examined the affliction of Cu/Zn-SOD expression with the GSH synthesis in the cells of *S. cerevisiae*, an important aspect not yet addressed in previous studies.

Materials and methods

Introduction of GAPDH promoter to pYES2.0

The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter from *S. cerevisiae* genomic DNA was amplified by polymerase chain reaction (PCR) using two ligonucleotide primers, a sense primer 5'-ACGGCCGG-CCTCGAGTCGAGTTTATCATTATCAATACTCG-3' and an antisense primer 5'-ACGGAGCTCGGATCCTTTATTTATGTGTGTTTATTCG-3' (the underlined represented *Nae*I and *Sac*I sites, respectively). The result-

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ing PCR fragment was digested with *Nae*I and *Sac*I and gel-purified before ligation into the same sites of the pYES2.0 vector and after removing the GAL1 promoter. The reconstructed pYES2.0 vector containing GAPDH promoter refers to as pGPD according to Yoo et al. (1999). Green fluoresce protein (GFP) was introduced to the pGPD, the pGPD-GFP for determining the activity of the GAPDH promoter. The promoter activity was observed after GFP expression in the cells of *S. cerevisiae*.

Clone and construction of Cu/Zn-SOD in pGPD

Encoding region of the Cu/Zn-SOD (accession No. DQ058108) was amplified by PCR with oligonucleotide primers, a sense primer 5'-ACGGAGCTCATGGTGAAGGCTGTTGTTGTGC-3', a antisense primer 5'-ACGAAGCTTTCAGCCTTGAAGTCCGATGATC-3' (the underlined represented *Nae*I and *Sac*I sites, respectively). The resulting PCR fragment was digested with *Sac* I and *Hind* III and gel-purified before ligation into the same sites of the pGPD-GFP vector and after removing GFP, the pGPD-Cu/Zn-SOD vector. In this vector, the introduced Cu/Zn-SOD-encoding sequence was fused at its N-terminus with the GAPDH promoter and the CYC-terminator sequence. The vector was then electrically transformed into the cells of *S. cerevisiae* (*ura*3-52, *trp*1-289, *leu*2-3, *leu*2-112, *his*3-D1). After 30 min incubation at 30°C, 500 µL transformants were spread on an agar plate containing 2% glucose, 0.67% Yeast nitrogen base, 2% agar and supplemented with appropriate amino acids (Roche).

Northern blot analysis of the Cu/Zn-SOD transcription

Independent confirmation of Cu/Zn-SOD expression was obtained by Northern Blot analysis, using total RNA isolated from the control and transformed cell strains according to the yeast handbook (Stratagene). Total RNA (10 µg) separated from each preparation were detected with a DIG-labeled PCR probe against full-length Cu/Zn-SOD after blotting. The expression was then detected with CDP-Star (Roche) using ImageMaster VDS-CL system (Amersham Pharmacia).

Activity analysis of rice (*Oryza sativa,)* Cu/Zn-SOD crudely extracted from the cells of *S. cerevisiae* by native polyacrylamide gel

S. cerevisiae containing the pGPD-Cu/Zn-SOD were inoculated into a 5-mL selective medium and then shaken at 180 rpm, 30°C until OD_{600nm} reached 1.0. The cells were obtained by centrifugation and the pellet was washed once with distilled water. The pellet was then resuspended in a 0.3-mL lyse buffer containing 10 mM sodium phosphate (pH 7.8), 1.0 mM EDTA, 0.1% Triton, and 1.0 mM PMSF (phenylmethylsulfonyl fluoride). The resuspension was centrifuged again at 12 000 rpm for 5 min. About 0.025 g of the cell pellet were disrupted by vigorous vortexing with glass bead (0.45–0.50 mm Sigma) for 5 min. Soluble proteins including the Cu/Zn-SOD were remained in the supernatant after centrifugation at 12 000 rpm for 5 min. The Cu/Zn-SOD activity was determined by the NBT (nitroblue tetrazolium) method. 10 µL of soluble proteins were loaded on a 12.5% native polyacrylamide gel. After electrophoresis, the gel was stained in a solution containing potassium phosphate (50 mM, pH 7.8), 275 mg/L NBT, 65 mg/L riboflavin and 3.2 ml/L TEMED (Tetramethylethylenediamine). The gel was placed in a glass tray and then illuminated with UV light for 5–15 min after 45-min dark incubation at room temperature. The gel became evenly blue during illumination except the light band containing Cu/Zn-SOD. Illumination was stopped at the point of maximum distinction achieved between the clear zone and the blue region. Crude extraction of the cells was analyzed by a 15% SDS polyacrylamide gels to confirm the molecular weight of the expressed Cu/Zn-SOD in *S. cerevisiae*.

SOD determination in *S. cerevisiae* cells

The determination of SOD activity in *S. cerevisiae* was described as Beauchamp and Fridovich (1971). One unit was defined as the amount of enzyme causing 50% decrease in the reduction of NBT under the test conditions. SOD activity was expressed as U/(10 mg) protein.

GSH determination in *Saccharomyces cerevisiae* cells

GSH determination was performed as the previous description by Fan et al. (2004). Cells were collected by centrifugation at 4000 × g for 5 min after the log phase and washed twice with distilled water, and then dried at 60°C to a constant weight for determining the biomass (He et al. 2000). About 0.05-g dry cells resuspended with 1-mL distilled water was stored at -20°C for 12 h. Then the frozen cells were incubated in boiling water for 5 min to release the GSH from the busted cells. The mixture was then cooled down to 25°C and centrifuged at 4000 × g for 5 min. The supernatant was used to detect GSH using DTNB (5,5′-Dithiobis(2-nitrobenzoic acid)) containing 0.5 mL of supernatant, 1.5-mL NaOH (0.15 M), 0.5-mL formaldehyde (3% v/v), 0.5-mL DTNB (100 mM) and 2.5-mL Tris/HCl (25 mM, pH 8). After 10 min incubation at 25°C, the concentration of GSH was determined at 412 nm wavelength and then calibrated with the SGH standard curve. The GSH was expressed as mg/g.

Results

Activity of GAPDH promoter

Strong green fluorescence from *S. cerevisiae* cells transformed with pGPD-GFP was detected by the fluorescence microscope (Fig. 1). In contrast, the fluorescence phenomenon was not found in cells of GFP absence, indicated by the cells transformed with pGPD only (data not shown). These observations together with the northern analysis (seen below) confirmed that the GAPDH activity was initiated functionally after the pGPD-GFP transformed into *S. cerevisiae* cells.

Northern blot, native polyacrylamide gel and SDS PAGE

Expression and activity of the Cu/Zn-SOD in the cells of the wild-type and transformed *S. cerevisiae* were determined by a Northern blot and a native polyacrylamide gel analysis, respectively (Fig. 2 a and b). The functional status of the Cu/Zn-SOD after expression in the cells of *S. cerevisiae* was confirmed by NBT after running a native polyacrylamide gel according to Hae Yong Yoo et al. (1999). The molecular weight of Cu/Zn-SOD was determined by a SDS-PAGE (Fig. 2c) with 16 KDa which is corresponding to the previous study demonstrated. (Hae Yong Yoo et al. 1999).

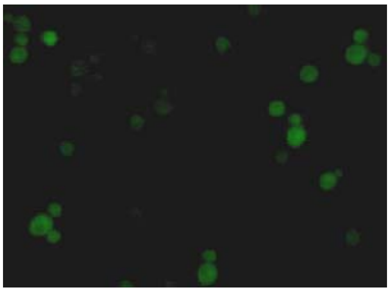

Fig. 1 GAPDH promoter activity confirmed by monitoring Heterologous GFP expression in *S. cerevisiae.* The GFP was transiently expressed in *wild-type S. cerevisiae,* and *S. cerevisiae* cells containing pGPD-GFP. Constructed plasmid was detected under UV light by applying FITC filter.

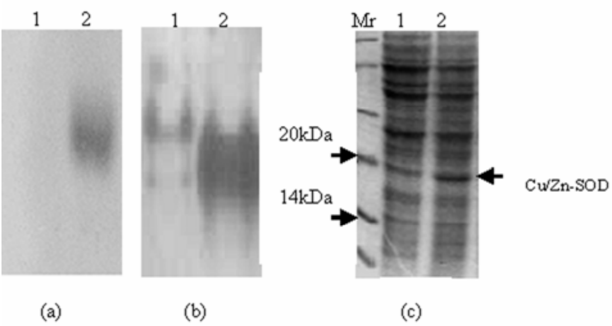

Fig. 2 Overexpression (a), activity (b) and molecular weight (c) of the Cu/Zn-SOD from the crude extraction of *S. cerevisiae* cells btained by using the analysis of Northern blot, Native gel and SDS-PAGE, respectively. Wild type (1) of *S. cerevisiae* was negative and the transformants containing pGPD-Cu/Zn-SOD (2) was positive. Markers size (Mr.), 20 kDa and 14 kDa.

Cu/Zn-SOD in *S. cerevisiae* cells

Relative low value of Cu/Zn-SOD was detected in the cells of wild-type *S. cerevisiae*. Cu/Zn-SOD expression in cells of the transformants was increased by three folds as compared with that in the wild type. Similar result was obtained in three independent experiments (Fig. 3a).

GSH synthesis in *S. cerevisiae* cells

GSH synthesis was determined independently in wild-type, pGPD-GFP and pGPD-Cu/Zn-SOD transformed *S. cerevisiae*. GSH value had a slight variation between the wild type and the pGPD-GFP transformed cells. Detected result showed less than 2% reduction of GSH synthesis in the pGPD-GFP transformed cells in comparison with the wild-type cells. However, GSH synthesis in the cells transformed pGPD-Cu/Zn-SOD was massively reduced down to the 60% of the value of wild-type cells (Fig. 3b). Similar result was also obtained after shifting the temperature up to 41°C for 1 h.

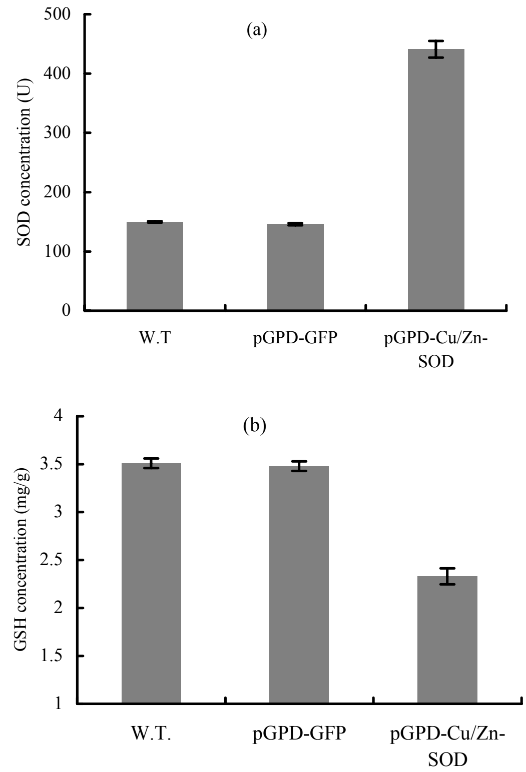

Fig. 3 Cu/Zn-SOD expression (a) and GSH synthesis (b) in non-transformed and transformed *S. cerevisiae* cells. The cells were grown at 30°C. The measurements of Cu/Zn-SOD expression were conducted at the point of OD_{600nm} reaching 1.0. W.T, non-transformed *S. cerevisiae cells*; pGPD-GFP, *S.cerevisiae* cells transformed with pGPD fused with GFP gene; pGPD-Cu/Zn-SOD, *S. cerevisiae* cells transformed with pGPD containing Cu/Zn-SOD gene.

Discussion

Cu/Zn-SOD, a major enzyme, involves in the reduction of superoxide anions from the cytoplasm (Jamieson et al. 1994). McCord and Fridovich (1969) demonstrated that Cu/Zn-SOD reacted with superoxide radicals to produce H_2O_2 and O_2. Also, GSH directly reacts with oxidant radicals to reduce them (Ishikawa et al. 1989). In this sense, Cu/Zn-SOD shares the compara-

ble function with GSH in removing ROS *in vivo*. The quantity analysis revealed that when the Cu/Zn-SOD was at predominant status in *S. cerevisiae*, the GSH synthesis was declined, and *vice versa*. Therefore, it can be inferred from the reveal that the transient expression of ectopic Cu/Zn-SOD gene in *S. cerevisiae* cells may limit the synthesis of endogenous GSH. However, no evidence was demonstrated that the affliction of the partially functionally overlapped genes was a phenomenon occurred generally in nature. Our result suggested that higher temperature increased the GSH synthesis in the cells of *S. cerevisiae* (data not shown), corresponding to the previous study that the ROS generated by a shift to a higher temperature activated the GSH1 and GSH2, which in turn would enhance GSH synthesis (Jamieson et al. 1998; Penninckx et al. 2000). It is uncertain whether the expression of Cu/Zn-SOD in *S. cerevisiae* cells may enzymatically restrain the GSH synthesis, possibly both GSH1 and GSH2 being responsible for the ectopic gene expression. Moreover, the cytosolic localization of the ectopic Cu/Zn-SOD gene expression may down-regulate GSH synthesis *in vivo*. The ROS may largely be reduced by Cu/Zn-SOD, but quantitatively not enough to motivate GSH synthesis, although the data exhibited an increasing response of the GSH synthesis to the higher temperature. In conclusion, the expression of Cu/Zn-SOD in the cells of *S. cerevisiae* may limit the endogenous GSH synthesis, sharing the overlapped function in response to definite stress, but more investigations are needed to demonstrate the important point in gene transformation.

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